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^{13}C High-Resolution Nuclear Magnetic Resonance Studies of Enzyme–Substrate Reactions at Equilibrium. Substrate Strain Studies of Chymotrypsin–*N*-Acetyltyrosine Semicarbazide Complexes

(kinetic study)

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ABSTRACT ***N*-Acetyl-L-tyrosine semicarbazide is hydrolyzed by chymotrypsin (EC 3.4.21.1) to *N*-acetyl-L-tyrosine and semicarbazide. If a high concentration of semicarbazide is present, the equilibrium for the reaction can be shifted from hydrolysis to synthesis. Using *N*-acetyl-L-[^{13}C]tyrosine enriched at the carboxyl carbon and high concentrations of semicarbazide hydrochloride, we have studied the enzyme–substrate complex of *N*-acetyl-L-[^{13}C]tyrosine semicarbazide and chymotrypsin A_δ by ^{13}C nuclear magnetic resonance. We observe no shift within the experimental accuracy of ±0.05 ppm as the fraction of substrate bound is changed from 0.17 to 0.70. Since E + S ⇄ ES is in fast exchange on the nuclear magnetic resonance time scale, it is possible to show that when the substrate is bound to the enzyme in the Michaelis complex, the ^{13}C resonance is shifted less than 0.1 ppm, indicating that negligible substrate strain occurs in this complex at the site of enzymatic attack. These experiments demonstrate the application of nuclear magnetic resonance to the study of particular states along the reaction pathway for enzyme–substrate reactions at equilibrium.**

A large majority of enzymatic reactions proceed by a series of several distinct steps that may include, besides the enzyme–substrate complex, covalent intermediates and metastable transition states. In order to understand how an enzyme functions and to assess the forces that contribute to its catalytic efficiency, one would like to compile detailed structural and kinetic data on each state in the reaction pathway. For certain well-studied systems like chymotrypsin an abundance of kinetic data has been assembled for the interaction of true substrates with enzymes. However, the methods for obtaining structural information, x-ray crystallography and high-resolution nuclear magnetic resonance (NMR), are not ordinarily suited for studying true enzyme–substrate complexes because of the long times required for data accumulation, during which the substrate is converted to product. Thus, except for the technically difficult experiment combining NMR and stopped-flow kinetics (1), these methods have been applied to systems composed either of active enzyme plus inhibitors or inactive enzyme plus true substrates (2–5). These results are often extrapolated to obtain information about an active enzyme–substrate complex.

The present report shows how it is possible to make high-resolution NMR measurements on an active enzyme–substrate system by a careful choice of reaction conditions. The principle of this method is to monitor the system under equilibrium conditions while forcing the equilibrium in the direction of the substrate with high product concentrations. To allow appreciable substrate binding, this requires that the product is not a competitive inhibitor. This permits lengthy NMR measurements while maintaining a constant high substrate concentration. Jencks *et al.* (6) demonstrated that in the reaction of *N*-acetyl-L-tyrosine hydroxamic acid with chymotrypsin, the equilibrium could be shifted from hydrolysis to synthesis simply by increasing the concentration of hydroxylamine, a hydrolysis product, in the reaction mixture. Recently Fersht and Requena (7) have extended these studies to a wide range of amides, including semicarbazide and formylhydrazide, as part of a detailed kinetic study of the chymotrypsin reaction mechanism (8). We have used the same strategy in the present study of complexes between *N*-acetyl-L-tyrosine semicarbazide and chymotrypsin.

A reaction mixture of chymotrypsin plus substrate at equilibrium will contain enzyme and substrate in four distinct states: (*1*) free in solution, (*2*) enzyme–substrate complex, (*3*) tetrahedral intermediate, and (*4*) acyl enzyme. The concentration of each species will be determined by the various equilibria constants and the initial concentrations of enzyme (E), substrate (S), and product (P_1)

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} ETI \underset{k_{-3}}{\overset{k_3}{\leftrightarrow}} EAc + P_1 \text{ (semicarbazide)}. \quad [1]$$

In theory, NMR is capable of examining each of these states. The information obtained from any particular NMR study is limited by the concentration of each species that can be maintained at equilibrium and the rates of conversion of one species to another. In the present experiments, the conditions allow us to obtain information about ES, the enzyme–substrate complex. The results show that the substrate, specifically *N*-acetyl-L-tyrosine semicarbazide, is not in a strained state when in the enzyme–substrate complex.

MATERIALS AND METHODS

Chymotrypsin A_δ (EC 3.4.21.1) (code-CDD) and chymotrypsin A_α (code-CDI) were purchased from Worthington Biochemical Corp. and used without further purification. Semicarbazide hydrochloride (ultra pure) was obtained from Aldrich Chemical Co. Proflavin was purchased from Nutritional Biochemical Corp., recrystallized several times

Abbreviation: NMR, nuclear magnetic resonance.

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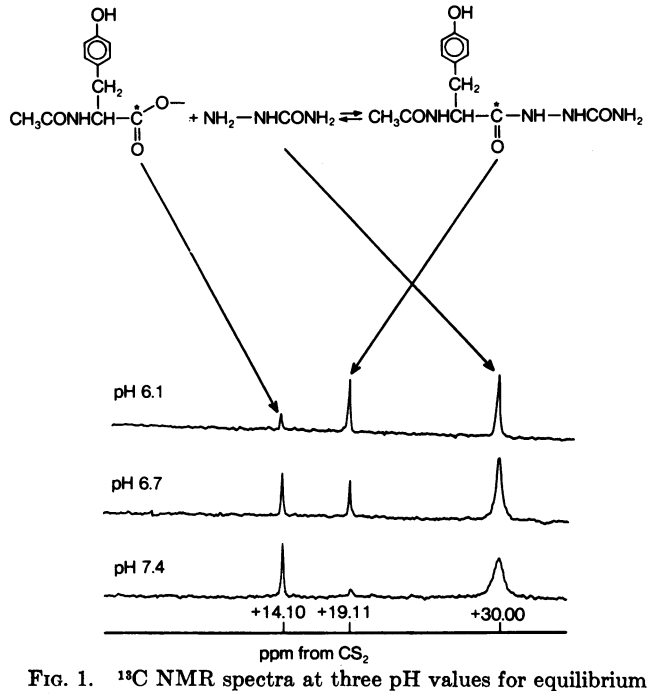

FIG. 1. ^{13}C NMR spectra at three pH values for equilibrium mixtures of chymotrypsin A_δ, *N*-acetyl-L-[^{13}C]tyrosine semicarbazide, *N*-acetyl-L-[^{13}C]tyrosine, and unenriched semicarbazide hydrochloride. The *asterisk* marks the enriched carbon. The spectra were taken at 7°C in 4 M semicarbazide hydrochloride, 0.1 M $CaCl_2$. The initial concentrations of enzyme and *N*-acetyl-L-tyrosine were 4 mM and 20 mM, respectively.

from water, and stored in the dark. *N*-Acetyl-L-tyrosine was purchased from Cyclo Chemical. Sodium [^{13}C]cyanide, 62% atom excess, was purchased from Merck, Sharp and Dohme of Canada. All other reagents were of the best commercial grade.

Synthesis of N-Acetyl-L-[carboxy-^{13}C]tyrosine. Sodium [^{13}C]-cyanide (62% atom excess) was used in the synthesis of DL-[*carboxy*-^{13}C]tyrosine through the intermediate hydantoin (9). *N*-Acetyl-DL-[*carboxy*-^{13}C]tyrosine ethyl ester was then prepared in crystalline form by the method of Niemann and McCasland (10). Enzymatic resolution was carried out by adoption of a published procedure (11). The racemic ester (2 g) in 16% (v/v) methanol (120 ml) was treated with chymotrypsin (4 mg) at pH 8 by addition of 1 M NaOH as needed. When the reaction stopped, the mixture was concentrated to about 20 ml under reduced pressure, and crystallization of the D ester was allowed to proceed. The ester was collected by centrifugation. The supernatant solution was combined with an aqueous washing of the precipitate and extracted with ethyl acetate to ensure removal of the ester. The aqueous layer was adjusted to pH 3 with 1 M HCl and lyophilized. The residue was stirred with several portions (10 ml) of acetone at room temperature. The extracts were clarified by centrifugation and concentrated in a stream of dry nitrogen to provide L-acetyl-[*carboxy*-^{13}C]tyrosine in quantitative yield as a glass.

Synthesis of N-Acetyl-L-tyrosine Semicarbazide was carried out by the mixed anhydride procedure (12). The crystalline product was isolated in 55% yield: melting point 197–199°, $[\alpha]D = +32°$.

Active Site Determination. *N*-Benzyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester (Schwarz/Mann) was used as an active site titrant in determining the number of active sites in the samples used in the NMR experiments. Fastrez and Fersht (13) have recently shown that this procedure underestimates the active site concentration in chymotrypsin A_δ by about 20% when compared with the *trans*-cinnomoyl-imidazole method. Thus, a 20% correction was applied to the values obtained by the *N*-benzyloxycarbonyl-tyrosine *p*-nitrophenyl ester method. After each NMR experiment, the sample was removed from the tube and dialyzed exhaustively for 36 hr against 1 mM HCl at 5°C. The active site titrations were carried out on these samples. In this manner any autolysis that occurred during the course of the experiment could be determined. From the value obtained at the end of the experiment, the minimum value for the active site concentration is obtained. It is this value that is used to compute the ratio of bound to total substrate in Fig. 3.

Dissociation Constants. The dissociation constant for the binding of *N*-acetyl-L-tyrosine semicarbazide to chymotrypsin A_δ was determined under the conditions of the NMR experiments by the proflavin displacement method (14). The experimental solutions were 4 M in semicarbazide hydrochloride, 0.1 M in $CaCl_2$, pH 6.1, $\mu = 1.4$. In this solution, K_D for proflavin and *N*-acetyl-L-tyrosine semicarbazide binding to chymotrypsin A_δ are 4.31×10^{-5} M and 1.08×10^{-3} M, respectively, at 25°C.

NMR Measurements. ^{13}C NMR measurements were recorded at 23.4 kGauss on a modified Varian XL-100 pulsed Fourier transform spectrometer equipped with a Nicolet 1080 digital computer for signal accumulation and Fourier transformation (15). All spectra were recorded using broad-band proton decoupling and 4K readout points. Transients were accumulated with a 1-sec interval between pulses. All resonance field positions are reported relative to field position of external $^{13}CS_2$.

RESULTS

Fig. 1 shows the chemical reaction studied in the presence of 4×10^{-3} M chymotrypsin A_δ. The initial concentration of ^{13}C-enriched *N*-acetyl-L-tyrosine was 20×10^{-3} M and of semicarbazide 4 M. In the spectra shown in Fig. 1, one can clearly resolve and identify the ^{13}C resonance positions of the free acid, the *N*-acetyl-L-tyrosine semicarbazide substrate, and semicarbazide hydrochloride. It must be emphasized that the concentration of semicarbazide hydrochloride is 10^3 times greater than that of the total ^{13}C-enriched *N*-acetyl-L-tyrosine in solution, which accounts for the high intensity of the unenriched semicarbazide resonance. A comparison of the three spectra indicates a strong pH dependence of the equilibrium. Since the objective of this experiment is to force the equilibrium as far to the left (Eq. 1) as possible, it is necessary to work around pH 6, where more than 80% of the tyrosine-containing reactants are in the substrate form. The extent to which the equilibrium is displaced to the left should also depend upon the concentration of semicarbazide. Fig. 2 shows a plot of the percent of *N*-acetyl-L-tyrosine semicarbazide at equilibrium relative to total initial *N*-acetyl tyrosine as a function of the semicarbazide hydrochloride concentration. The relative concentrations of *N*-acetyl-L-tyrosine and *N*-acetyl-L-tyrosine semicarbazide are determined directly from the areas under the resonance peaks in the spectra. It is clear from the data shown in Figs. 1 and 2 that high concentra-

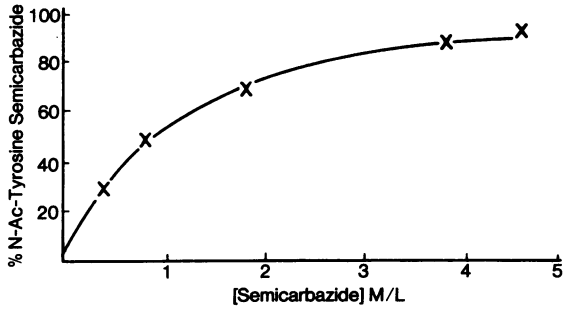

FIG. 2. Plot of the percentage of *N*-acetyl-L-tyrosine semicarbazide at equilibrium as a function of semicarbazide hydrochloride concentration. The reactions were carried out at 7°C, pH 5.7, in solution with ionic strength about 1.3. The synthesis was catalyzed by chymotrypsin A_δ (3 to 4 $\times$ 10^{-3} M). The initial *N*-acetyl-L-tyrosine concentration was 15 $\times$ 10^{-3} M. Ratios of *N*-acetyl-L-tyrosine to *N*-acetyl-L-tyrosine semicarbazide were determined by the areas under the NMR resonance peaks.

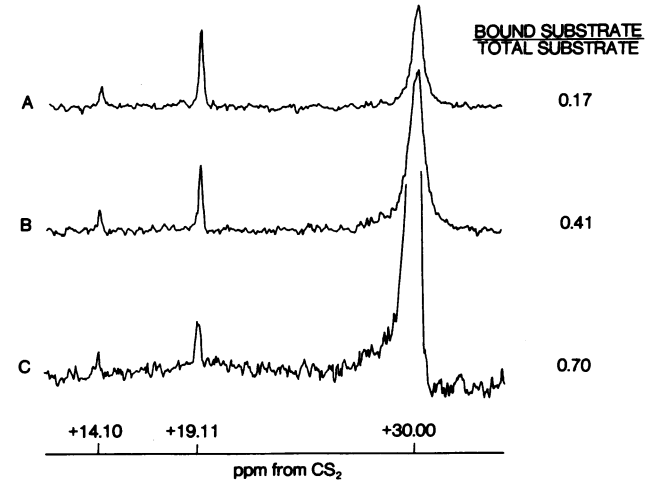

FIG. 3. ^{13}C NMR spectra of solutions of chymotrypsin, *N*-acetyl-L-[^{13}C]tyrosine, and semicarbazide hydrochloride. The initial concentrations of *N*-acetyl-L-tyrosine were 20.4, 7.6, and 1.9 $\times$ 10^{-3} M in (*A*), (*B*), and (*C*), respectively. The initial enzyme concentration was maintained constant at 3 $\times$ 10^{-3} M. The spectra were taken at pH 6.1, 7°C in 4.0 M semicarbazide hydrochloride, 0.1 M $CaCl_2$, ionic strength about 1.4. The number of transients are 4,000, 8,000, and 65,000 for (*A*), (*B*), and (*C*), respectively. The relative intensities at +19.11 and +14.10 ppm showed that 81% of the ^{13}C-enriched tyrosine is present as *N*-acetyl-L-tyrosine semicarbazide. The active site concentrations were measured at the end of each run. The amounts of substrate bound were calculated from these enzyme concentrations using the dissociation constant K_D = 1.08 $\times$ 10^{-3} M, determined for these solutions by the proflavin displacement method.

tions of the substrate can be maintained at equilibrium in the presence of the enzyme, simply by using high concentrations of the semicarbazide product at about pH 6.

By selection of favorable conditions, as indicated from the above results, the binding of *N*-acetyl-L-tyrosine semicarbazide to chymotrypsin was studied at pH 6.1. Fig. 3 shows three spectra for a solution of enzyme, substrate, and products at equilibrium. The compositions of the solutions, including pH and ionic strengths, are identical for each spectra except that the total concentration of *N*-acetyl-L-tyrosine is varied. As the total amount of *N*-acetyl-L-tyrosine, and consequently *N*-acetyl-L-tyrosine semicarbazide, is decreased, the ratio of bound to total substrate increases. This ratio (listed on the *right* of each spectrum in Fig. 3) was calculated from the measured value of K_D = 1.08 $\times$ 10^{-3} M. The spectra show the same positions and the same relative intensities for the resonance of the substrate at the three ratios. For the greatest accuracy, we refer the spectra of Fig. 3*B* and *C* back to Fig. 3*A*, in which the fraction of substrate bound is very small. Given a signal to noise ratio of about 8:1 for the *N*-acetyl-L-tyrosine semicarbazide resonance at pH 6.1 (see Fig. 3*C*) and a linewidth of 8 Hz, the absence of an observable resonance shift means that any shift that occurs must be <1 Hz $\equiv$ 0.04 ppm. The higher concentration of substrate used in Fig. 3*B*, which also shows no shift from Fig. 3*A*, gives a better signal to noise ratio, which allows us to put a limit of $\pm$0.02 ppm upon its shift.

DISCUSSION OF RESULTS

These results must be discussed in terms of the states that are present at equilibrium, as given in Eq. 1. To begin, we must determine which steps of this reaction pathway are responsible for the observed resonances. From Fig. 3 we see that an appreciable fraction of substrate is free in all three measurements. Hence, a large part of the signal at +19.11 ppm comes from free substrate, but it is not certain whether it includes contribution from the enzyme–substrate complex. For this to be so the molecules must be in fast exchange between the free state and the enzyme–substrate complex. The criterion for fast exchange is that the lifetime, τ, of the ^{13}C nucleus in either state must be short compared to the reciprocal of the NMR frequency shift between these two states, or $1/\tau \gg 2\pi\Delta\nu$. An upper limit of the ^{13}C chemical shift that might be introduced by the strains of binding is estimated to be about 4 ppm by considering the relative shifts of homologous carbonyl carbons in five-, six-, and seven-membered cyclic ketones (16, 17). Hence, for fast exchange it is necessary that, for at least one of the states, $\tau \ll 1.6 \times 10^{-3}$ sec. Hess *et al.* (18) have determined k_1 and k_{-1} to be approximately 10^6 M^{-1} sec^{-1} and 10^3 sec^{-1}, respectively. With 3 $\times$ 10^{-3} M enzyme, k_1 becomes 3 $\times$ 10^3 sec^{-1}. Thus, if the shift between free substrate and the enzyme–substrate complex were as large as 4 ppm, the conditions for fast exchange would not be fulfilled. As shown in Fig. 1, there is a mixture of 20% *N*-acetyltyrosine and 80% *N*-acetyltyrosine semicarbazide at equilibrium at pH 6.1 in 4 M semicarbazide. Since *N*-acetyltyrosine binds about 100 times weaker than *N*-acetyltyrosine semicarbazide [K_i for *N*-acetyltyrosine = 1.15 $\times$ 10^{-1} M (19)], the amount of *N*-acetyltyrosine bound relative to bound substrate is negligible. Therefore, the intensity of the *N*-acetyltyrosine resonance (+14.10 ppm) in each spectrum in Fig. 3 may be used as an internal standard for measuring the intensity of the *N*-acetyltyrosine semicarbazide resonance. When the intensity ratios for these two resonances are compared, it is seen that they are constant for all three spectra in Fig. 3, to within the experimental accuracy of measuring intensities, i.e., $\pm$10%. There is no loss in signal intensity of the +19.11 ppm resonance as the ratio of bound to total substrate increases. We must conclude, therefore, that the +19.11 ppm resonance includes contributions from both the bound and free forms of substrate. Since the resonance positions are identical for free *N*-acetyltyrosine semicarbazide and the substrate in the enzyme–substrate complex within an experimental accuracy of $\pm$0.05 ppm, we can con-

clude that the shift in the enzyme–substrate complex is < 0.1 ppm. This conclusion suggests that any strain occurring at the peptide bond in the enzyme–substrate complex must be orders of magnitude smaller than those introduced by going from five- to six-membered cyclic ketones. It is worth noting that the dipolar interaction with the nearest proton would produce a negligible broadening of the ^{13}C resonance in the enzyme–substrate complex (20), which explains why we do not observe any broadening.

It should be noted that these experiments have been performed at pH 6, where the imidazole group is protonated and the enzyme is inactive. It is conceivable that at pH 7–8, where the enzyme is active, the substrate might bind in a slightly different orientation and substrate strain might occur. However, the pH independence of K_i for neutral inhibitors observed by Johnson and Knowles (21) suggests that this situation should not occur.

We must consider whether substrate species further down the reaction pathway are contributing measurable signal intensity to the observed resonance or altering the resonance field position. From ^{13}C resonance positions of amides and hemiacetals we estimate that the resonance position of the tetrahedral carbon should be about 40 ppm downfield from the amide or the enzyme–substrate complex. For fast exchange between enzyme–substrate and tetrahedral intermediate, either k_2 or k_{-2} must be $\gg 6 \times 10^3$ sec^{-1}. At low pH, k_2 is rate limiting in the hydrolysis of *N*-formyl phenylalanine semicarbazide with a value of 0.04 sec^{-1} (22). We do not expect this to be appreciably different for the present substrate. Hence, the only way enzyme–substrate complex $\rightleftarrows$ tetrahedral intermediate could be fast on the NMR time scale is for $k_{-2} \gg 6 \times 10^3$ sec^{-1}. Such a rate cannot be excluded *a priori* from existing measurements. However, if k_{-2} were $\gg 6 \times 10^3$ sec^{-1}, then the ratio of tetrahedral intermediate to enzyme–substrate complex at equilibrium would be k_2/k_{-2} or $\ll 10^{-5}$. If only 1 in 10^5 of the substrate were present as tetrahedral intermediate, the shift would be 40×10^{-5} ppm, which is too small to be observed. Thus, at equilibrium the only significant substrate species present in a mixture of chymotrypsin and *N*-acetyltyrosine semicarbazide are free substrate and the enzyme–substrate complex. There will be no measurable contribution from other substrate species further down the reaction pathway.

The particular advantage of this present approach is that one may be able to select different substrates whose equilibria constants vary so that different intermediate states may be examined. For example, *N*-acetyltyrosine formylhydrazide has reaction rates that suggest that, under conditions similar to those used here, 10% of the substrate might be present as the tetrahedral intermediate in slow exchange with enzyme–substrate complex. Therefore, a separate resonance for tetrahedral intermediate should be observed that should not be broadened either by kinetics or by binding to the enzyme.

In summary, these experiments demonstrate the ability of NMR to study isolated states in an enzymatic reaction pathway when the reaction mixture is at equilibrium.

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